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MICROBIOLOGICAL PRODUCTION OF GEOSMIN

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16. ABSTRACT

Geosmin, trans-1, 10-dimethyl-trans-9-decalol, is produced by numerous actinomycetes and some blue-green algae. It has been shown to be responsible for earthy/musty odors in public water supplies. Samples of purified geosmin were needed by the U.S. Environmental Protection Agency and by others for use as odor standards and for use in water treatment unit process development studies. This work was carried out to fulfil this need. The microorganism, Streptomyces sp. CWW3, was grown for 5 days at 28° with shaking in a broth medium. The mixture was steam distilled and the clear distillate extracted with methylene chloride. The geosmin containing methylene chloride extract was separated and the geosmin purified by column and gas chromatography. Seven hundred nine milligrams of geosmin was prepared. This report was submitted in fulfillment of Research Grant No. R-801630 by the Institute of Microbiology, Rutgers - The State University of New Jersey under the sponsorship of the Environmental Protection Agency. Work was completed as of June 15, 1974.

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment -- air, water, and land. The National Environmental Research Centers provide this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on man and biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

This research has provided increased understanding of aspects of the microbiological production of a chemical compound which causes objectionable tastes and odors in water supplies, and has resulted in the availability of standard samples of that compound. This new understanding and the samples themselves can now be used by other researchers in monitoring of water treatment unit processes and development of appropriate new control technology.

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Abstract

Geosmin, trans-1, 10-dimethyl-trans-9-decalol, is produced by numerous actinomycetes and some blue-green algae. It has been shown to be responsible for earthy/musty odors in public water supplies. Samples of purified geosmin were needed by the U.S. Environmental Protection Agency and by others for use as odor standards and for use in water treatment unit process development studies. This work was carried out to fulfil this need.

The microorganism, Streptomyces sp. CWW3, was grown for 5 days at 28° with shaking in a broth medium. The mixture was steam distilled and the clear distillate extracted with methylene chloride. The geosmin containing methylene chloride extract was separated and the geosmin purified by column and gas chromatography. Seven hundred nine milligrams of geosmin was prepared.

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Conclusions

It is possible to prepare pure geosmin in milligram amounts from *Streptomyces* sp. CWW 3.

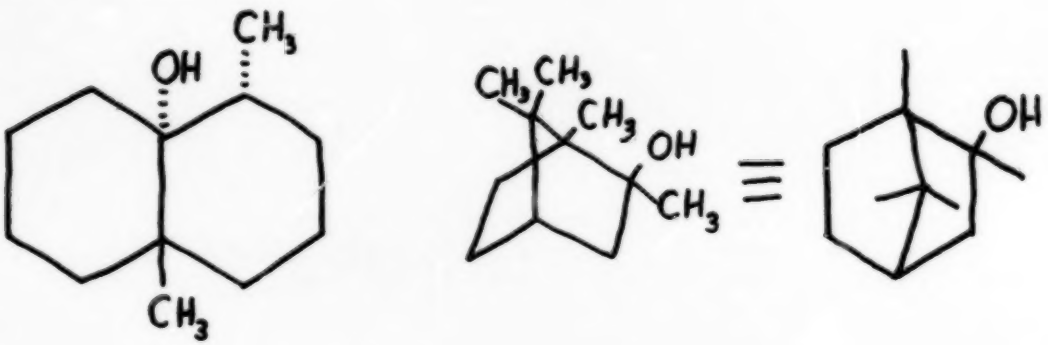
Recommendations

Milligram or gram amounts of geosmin could probably be produced by any one of the American pharmaceutical companies who presently make antibiotics by fermentation from streptomyces.

Introduction

Geosmin from the Greek "geos" meaning earth and "osme" meaning odor is the name which has been given to the substance responsible for the characteristic "earthy" odor of the soil. It is produced by numerous actinomycetes[1,2] and some blue-green algae[3,4]. Geosmin and methylisoborneol[5,6] (Fig. 1) have been shown to be responsible for earthy/musty odors in public water supplies in North America[6], Europe[7], and Japan[8]. The threshhold odor of geosmin is 0.1 ppb[3]. It is probably geosmin which accumulates in the flesh of fish which inhabit earthy/musty tainted water[9-11] making the fish unpalatable and unmarketable.

Since our initial isolation[1] and structure determination[12] of geosmin as trans-1, 10-dimethyl-trans-9-decalol we have had many requests for authentic samples of geosmin. Twenty three requests from 1967-1972 were listed in our grant application. The majority of the requests involved research on water or fish. Since then our supplies of geosmin have been exhausted. Because of the number of requests received by us and by EPA, financial assistance was provided to our laboratory by EPA for production of purified geosmin to be distributed to other researchers by EPA and used in EPA water treatment unit process development studies.



geosmin methylisoborneol

Fig. 1. The structures of geosmin and methylisoborneol.

The yields of isolated geosmin have always been about 1 mg per liter of whole broth. This meant that the typical 40 flask, 10 l batch which is the largest that a technician can conveniently handle yields about 10 mg of geosmin. A reasonable production schedule considering the amount of shaker space available at 28° for the 2 l production flasks is one batch per week. The preparation of seed cultures and media, sterilization and innoculation as well as work up of the batch takes about half the work time of one technician.

Therefore, we contracted to prepare 10 mg of geosmin per week after a start-up delay of 1 month. This suggests 480 mg of geosmin as the year's output. Because during the year we were able to increase the yields slightly, we actually sent 709 mg of pure geosmin in four quarterly shipments. We also sent pure methylisoborneol (16 mg) and some mixtures of geosmin and methylisoborneol (71 mg).

Experimental

Sterilization. Normal sterilization procedure is in an autoclave 20 min. at 121° using 15 pounds steam pressure.

Media. The recipes given are for liquid media (broth). For solid media add 15 g agar per liter. Cerelose is a commercial grade of glucose.

Soy-Bean. 10 g Staley's soybean meal, 20 g Cerelose, 5 g Bacto-peptone or 10 g Wilson Co. meat peptone 851C, 5 g sodium chlorine per liter of tap water, pH adjusted to 7.5 with 40% aqueous NaOH before sterilization, about 500 mg of $CaCO_3$ added to each 2 liter flask.

Yeast-Dextrose. 10 g Difco yeast-extract, 10 g Cerelose per liter of tap water, pH adjusted to 7.0-7.2 with 40% aqueous NaOH.

Gas Chromatography (GC). GC was carried out with an F & M model 700 dual column instrument with a thermal conductivity detector. The coiled copper column was 6 ft by 1/8 inch and contained 10% SE-30 in Diatoport W, 60-80 mesh. This is a non-polar column which separates substances roughly in order of the volatility. Carrier gas was helium at 50 ml/min.

Actinomycetes in Sterile Soil. To 100 g of garden soil which has been autoclaved for 1-2 hours is added 1 g $CaCO_3$ and 259 mg. of dried blood. Next day the mixture is autoclaved again for 1-2 hours, and then 20 ml sterile distilled water and about 4 ml of packed cells is added. The cells are obtained by centrifuging a freshly grown broth culture and are usually washed with sterile water. The soil-cells mixture is incubated for 2-3 days at 28° then stored in the refrigerator.

Production of Geosmin. A small portion of soil containing Streptomyces sp CWW3 was taken with a sterile spatula and innoculated into yeast-dextrose broth, 50 ml/250 ml flask. After 24 hours incubation at 28° on a rotary shaker at about 200 rpm, the resulting cell suspension was pipetted aseptically into 14 similar flasks of yeast-dextrose broth (2 1/2 ml of cell suspension per flask). After 24 hours shaking at 28° these flasks were used to innoculate 40 flasks of soy-bean medium, 250 ml/2 liter flask (1/3 of the contents of a small flask poured aseptically into each large flask.) After 5 days on a reciprocal shaker, 80 strokes per min. at 28° the contents of the flasks were combined and distilled at atmospheric pressure until about 2 liters of distillate had collected. Boiling chips and several milliliters of polyglycol-2000 antifoaming agent were added to minimize foaming (Hodag Chem. Co., Chicago, Ill. or Dow Chemical Co., Midland, Mich.). The distillate was extracted twice with 10-20% of methylene chloride and the methylene chloride extracts concentrated in a stream of warm air. The extracts of the distillate from one such 10 liter batch after concentration to 10 ml was suitable for gas chromatographic (hereafter called GC) assay and was stable to storage at 5°. Typical yields, deduced from the areas of the peaks in the GC assay were: methylisoborneol 16 mg, geosmin 23 mg and sesquiterpenol 13 mg (hereafter called MIB, geos and SQ).

Table 1. RETENTION TIMES OF MIB AND GEOS

Initial temp.	Rate of temp. increase	Retention time (min.) of MIB	GEOS	SQ
80°	5°/min	14	20.5	27
100°	"	10	16	21.5
120°	"	7	12	18

Purification of Geosmin. In a typical purification experiment the distillate-extract concentrate from 2 batches was further concentrated and applied to a chromatography column made up of 'Baker, analyzed' reagent aluminum oxide powder (12g) in methylene chloride. The aluminum oxide had previously been stirred several hours with distilled water, filtered, and air dried for several days at room temperature. The 3 desired volatile alcohols passed readily through the alumina column and were detected in the first three 10 ml eluate fractions, but acidic and dark impurities were retained on the column. The 3 eluate fractions were combined and applied to a narrow (1.4 x 36 cm) 20 g column of 'Baker analyzed' reagent silica gel powder in methylene chloride. Elution was with methylene chloride, 10 ml fractions. GC assay revealed the products, and the amounts were estimated from peak areas.

The retention times of the 3 volatile products are given in Table 1. The results of a typical purification experiment are summarized in Table II.

Table II. SUMMARY OF SILICA GEL CHROMATOGRAPHY

Fraction #	Total Vol	Product and Amount
1-3	60	nothing
4	10	35 mg geos.
5	10	21 mg geos & 7 mg MIB
6	10	21 mg MIB
7	10	10 mg MIB
8-12	50	42 mg SQ

Subsequent purifications of up to ten combined batch extracts were carried out in exactly the same way with appropriate increases in amounts of reagents.

Preparation of Samples. Three types of samples were prepared.

Mixed Geosmin and Methylisoborneol. Fractions #4-7 (Table II) were combined then dispensed into 3 glass ampoules. The methylene chloride solvent was removed in a stream of air and the last traces of solvent were removed by placing the ampoules in a vacuum oven for 2 minutes at 25-27 inches vacuum. Although fractions 4-7 assayed 94 mg of geosmin and MIB the weight in the 3 ampoules after solvent removal was 71 mg of this mixture. This loss of the desired volatile products during solvent removal was unadvoidable and was sometimes as much as 50%.

Purified Geosmin. In later experiments eluate fractions containing geosmin only, were used and 70% ethanl was chosen as the solvent for shipment. Most ampoules were shipped containing 20-35 mg geosmin and 0.5 ml 70% ethanol, and the exact weight of geosmin was recorded on each ampoule. The 70% ethanol solvent was chosen because it preserved the sterility of the sample without dehydration. This solvent was also compatible with the aqueous phase in eventual dilution for studies in water.

GC Pure Geosmin and MIB. In another experiment, the relevant eluate fractions from the alumina column were taken to dryness and redissolved in a small volumn of cyclohexane for preparative GC. In solution were 16 mg MIB, 23 mg geosmin and 13 mg SQ, but the amounts recovered from the preparative GC were 8 mg MIB, 15 mg geosmin and 8 mg SQ. These losses during preparative GC were typical and unavoidable under the conditions used. GC pure materials were collected in capillary tubes 1.6 - 1.8 x 100 mm which fit snugly into the exit port of the GC. The tubes were sealed at both ends before shipment.

Summary of Samples Sent. A summary of samples sent to EPA is presented in Table III.

Table III. SUMMARY OF SAMPLES SENT TO EPA

Date	Samples	Amount and Form
9/28/73	geos.	20 small sealed tubes each 1.5 mg
	MIB	20 small sealed tubes each 0.8 mg
10/1/73	geos & MIB mixture	25 mg in 1 ml H_2O & 3 drops $CHCl_3$, sealed ampoule
		21 mg in 70% ethanol, sealed ampoule
		25 mg no solvent, sealed ampoule
11/1/73	geos.	10 sealed ampoules total 184 mg
3/5/74	"	10 sealed ampoules total 308 mg
6/20/74	"	5 sealed ampoules total 187 mg

Total geosmin 709 mg

Discussion

Streptomyces sp CWW3 had been isolated from Lake Michigan water. It was received from Miss Lilia McMillan of the Central Water Filtration Plant, Chicago, Illinois and was considered, along with several other strains which were sent at the same time, to be implicated in odor problems for that department. In order to preserve this culture with the minimum of change it was immediately put into sterile soil. Frequently when a micro-organism is taken from its natural environment and placed on laboratory media it ceases to produce the substance or feature which one desires to investigate. However, CWW3 stored in soil, during 3 years was a stable and reliable producer of geosmin, methylisoborneol and the sesquiterpene alcohol $C_{15}H_{26}O$. Other geosmin producing actinomycetes are listed in references 1 and 2, however, it should be emphasized that many of them were tried only once. In the cases where repeated fermentations were done, the yields of geosmin were variable and low.

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